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Distribution of erythropoietin producing cells in rat kidneys during hypoxic hypoxia

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Distribution of erythropoietin producing cells in rat kidneys during hypoxic hypoxia. We have used *in situ* hybridization to determine the localization and distribution of cells expressing the erythropoietin (EPO) gene in kidneys of rats exposed to reduced oxygen tensions to characterize the control of renal EPO formation during hypoxic hypoxia. Animals were subjected to severe hypoxia (7.5% O_2) for 4, 8 and 32 hours to assess changes related to the duration of hypoxic exposure, and additionally to 9% and 11.5% O_2 for eight hours to define changes related to the degree of hypoxia. The number of cells containing EPO mRNA were counted on tissue sections and compared to tissue concentrations of EPO mRNA and to the serum hormone concentrations. *In situ* hybridization revealed expression of the EPO gene exclusively in peritubular cells that were predominantly located in the cortical labyrinth under all conditions tested. After four hours of severe hypoxia (7.5% O_2) approximately 170-fold more cells were found to contain EPO mRNA than under normoxic conditions. The number of EPO producing cells did not change significantly between four and eight hours exposure to 7.5% O_2, but the amount of EPO mRNA per kidney increased approximately threefold. Further continuation of hypoxia resulted in down-regulation of renal EPO mRNA levels, which was mainly due to a reduction in the number of cells containing EPO mRNA. Comparison of graded degrees of hypoxia applied for eight hours showed an inverse exponential relationship between oxygen tension and the number of EPO producing cells. This recruitment of cells expressing the EPO gene occurred along a gradient extending from the corticomedullary border to the subcapsular tissue. In view of previous observations in anemic animals these findings indicate that the control of EPO formation under anemic and hypoxic hypoxia is similar. The cell type producing EPO appears to be identical and the number of cells expressing the EPO gene appears to be a major determinant of EPO production rate under both conditions. The observation that recruitment of EPO producing cells is reversed during prolonged continuous hypoxia seems compatible with both increases in tissue oxygenation as well as cellular adaptation to hypoxia.

The glycoprotein hormone erythropoietin (EPO) is the major regulator of red cell formation [1]. In adults it is mainly produced by the kidneys, and production is increased in response to two main stimuli: reduction in oxygen carrying capacity (anemic hypoxia) and reduction in arterial oxygen tensions (hypoxic hypoxia). A major control of EPO formation has been shown to operate through regulation of the level of its mRNA [2–4]. Experiments with isolated perfused kidneys have shown that oxygen sensing mechanisms exist within the kidney itself [5–7]. Precisely how changes in blood oxygen availability arising from anemic or hypoxic hypoxia affect intrarenal oxygenation and regulate expression of the EPO gene is, however, still unclear.

Although anemic and hypoxic hypoxia both effectively stimulate EPO formation, there are differences, and a number of observations suggest that the regulation of EPO formation under the two conditions may not be identical. Basically, anemic and hypoxic hypoxia differ in that during anemia a measurable reduction in oxygen tension occurs only after extraction of oxygen from arterial blood, and putative oxygen-sensing mechanisms are thus not only affected by oxygen supply, but depend essentially on oxygen consumption. Second, regarding the site of the "oxygen sensor", experiments with isolated perfused kidneys have shown that EPO mRNA levels [5] and EPO secretion [6, 7] increase upon lowering the oxygen tension of the perfusate, indicating that hypoxic hypoxia is sensed intrarenally. However, varying the hematocrit of the perfusate was not found to influence EPO synthesis of isolated perfused kidneys, and it was therefore suggested that under anemic hypoxia, extrarenal signals may play an important role in stimulating EPO formation [7]. Third, regarding the cellular site of EPO production within the kidney, Koury, Bondurant and Koury [8] and Lacombe et al [9] detected EPO mRNA in peritubular cells of the renal cortex of anemic mice using *in situ* hybridization. Similar results were obtained in anemic rats [10], and in accordance with these findings, EPO mRNA was also detected in interstitial cells of human polycystic kidneys [11]. In contrast, Maxwell et al reported EPO production in renal tubular cells of animals subjected to hypoxic hypoxia [12], and the difference in their results may raise the possibility that different cells produce EPO, depending on the type of stimulus. Fourth, with respect to the overall ability to stimulate EPO formation, it is well established that severalfold higher serum EPO levels can be observed in response to anemia than under the most severe degrees of hypoxic hypoxia [1]. Fifth, regarding the kinetics of EPO formation, serum EPO

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levels during continued hypoxic hypoxia reach maximal values within about 18 hours in rodents [13–15] and 48 hours in humans [13, 16], but thereafter decline and are only moderately elevated during prolonged hypoxia. Also during chronic intermittent hypoxia (22 hr/day) most of the increase in EPO levels in mice was found to be transient [17]. In contrast, during chronic anemia EPO levels tend to remain elevated up to several thousandfold [1].

In view of these differences we felt that assessing the pattern of EPO gene expression during hypoxic hypoxia might provide further insight into oxygen dependent regulation of EPO. We have therefore used *in situ* hybridization to characterize renal EPO mRNA accumulation during hypoxic hypoxia and to compare it with previous observations in anemic animals [8–10]. We were first interested in defining the type and location of EPO producing cells during hypoxic hypoxia. Second, we wanted to know if, and under which conditions, variations in the renal amount of EPO mRNA during hypoxic hypoxia are due to changes in EPO mRNA levels of single cells or due to variations in the number and location of the cells expressing the gene. To this end rats were studied after exposure to varying degrees of hypoxia (11.5% O_2, 9% O_2, 7.5% O_2) for eight hours, a time point at which previous studies showed maximal production rates for EPO [15]. In addition, animals were studied after four and 32 hours of severe hypoxia (7.5% O_2), time points at which EPO production is not yet maximal, or has already declined from its peak [15]. The number of cells in the kidney containing EPO mRNA as determined by *in situ* hybridization was related to the total organ quantity of EPO mRNA as measured by RNAse protection in homogenates of the contralateral kidneys and to serum hormone levels of the animals.

Methods

Animals

Twenty-one adult male ZUR:SIV strain rats (body wt 268 ± 27 g, mean ± SD) were used.

Stimulation of EPO formation

Normobaric arterial hypoxia was used as the stimulus for EPO formation. To this end animals were exposed to atmospheres low in oxygen (7.5, 9, and 11.5% O_2) in a chamber gassed with appropriate mixtures of normal air and nitrogen. Groups of three animals were exposed to these stimuli for eight hours and three to six animals were in addition exposed to 7.5% O_2 for 4 or 32 hours. At the onset of experiments the chamber was flushed to achieve the respective oxygen tensions within approximately 25 minutes. Oxygen concentration of the gas mixture inside the chamber was checked by means of an Bacharach Fyrite oxygen indicator. Litter mates of the animals exposed to hypoxia were used for study of EPO formation under basal conditions.

Within 15 minutes of completion of hypoxic exposure animals were sacrificed by cervical dislocation. Blood from the abdominal aorta was removed for determination of serum EPO concentrations. Both kidneys were rapidly removed. Since previous studies have shown that EPO mRNA concentrations in paired kidneys of the same animals are similar [18], one kidney of each animal was used for *in situ* hybridization and the contralateral kidney homogenized for determination of the total organ quantity of EPO mRNA, to allow a direct comparison between both parameters. The kidney used for *in situ* hybridization was cut into four sections perpendicular to the longitudinal axis and immediately immersed in 4% neutral buffered paraformaldehyde and fixed overnight at 4°C. The contralateral kidney was homogenized in 24 ml of guanidine thiocyanate (4 M) containing sarcosyl (0.5%), EDTA (10 mM), sodium citrate (25 mM) and mercaptoethanol (700 mM). Tissue homogenates were frozen at −80°C until RNA was prepared.

Determination of EPO mRNA

In situ hybridization. Fixed kidney specimens were washed in tap water, dehydrated in ethanol and embedded in paraffin wax. The paraffin blocks, each of which contained the four pieces of kidney obtained from one rat were coded prior to further processing. Sections 3 μm in thickness were cut from each block and processed for *in situ* hybridization as previously described [19, 20].

Probes. A partial rat EPO cDNA was generated by the polymerase chain reaction using primer sequences based on the mouse EPO gene sequence [10]. The fragment was 482 base pairs in length and included all of the exons III and IV, parts of exons II and V and 20 base pairs of sequence corresponding to synthetic restriction enzyme sites. The cDNA fragment was cloned directly from the polymerase chain reaction mixture into the polylinker region of the plasmid pCR 1000 (Invitrogen, San Diego, California, USA). Clones were isolated that had the cDNA insert in either the sense or antisense orientation with respect to the T7 RNA polymerase promotor of the plasmid. Labeling of sense and antisense RNA was achieved using ^{35}S-UTP (850 Ci/mmol, Amersham International, Arlington Heights, Illinois, USA) during *in vitro* transcriptions performed with the Riboprobe Gemini II Core System (Promega Biotec, Madison, Wisconsin, USA). Prior to *in situ* hybridization limited alkaline hydrolysis was used to cut probes to an average length of 150 base pairs [21], which was confirmed by gel electrophoresis.

Quantitation of EPO producing cells. The numbers of EPO producing cells in the kidneys of rats subjected to the various experimental stimuli were quantitated as previously described [19]. The total number of EPO producing cells in each of the four pieces of kidney in each section were counted using the 20× objective on an Olympus BH-2 microscope. The counts were performed in a blind manner since sections were taken from blocks which were randomly coded prior to sectioning. The code was not broken until all sections were counted. Cells were considered to be positive if the density of silver grains over them, assessed by focusing up and down, was greater than that of the general background. After all sections were counted, the microscope slides were projected at a final magnification of 11×, and kidney outlines and corticomedullary boundaries were traced onto paper. Cortical area was determined by cutting and weighing the paper. Results were then expressed as the number of EPO producing cells/cm^2 of cortex. Quantitation of EPO producing cells was performed twice in independent hybridization experiments. Results of both experiments were closely correlated ($n_1 = -28.7 + 1.11 \times n_2$; n_1 and n_2 = number of EPO producing cells per square cm of renal cortex in the first and second experiment, respectively; $r^2 = 0.95$, $P < 0.0001$). Values presented subsequently are means of the number of

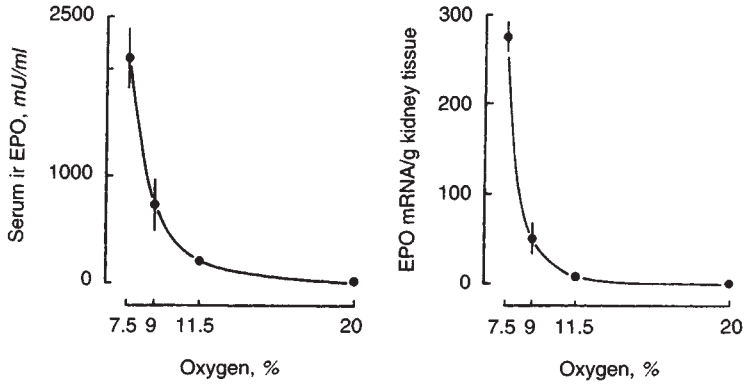

Fig. 1. *Serum immunoreactive erythropoietin (ir EPO) levels (left) and renal EPO mRNA concentrations (right) in animals studied under normoxic conditions or exposed to different degrees of hypoxia (7.5% O_2, 9% O_2, 11.5% O_2) for eight hours.* EPO mRNA was measured by RNAse protection, quantified by scintillation counting of protected EPO mRNA fragments and is expressed in arbitrary units relative to an external standard, that was coanalyzed with each assay. Values are mean ± SE, $N = 3$.

positively labeled cells in both *in situ* hybridization experiments of each kidney specimen.

RNAse protection assay. Total RNA was purified from kidney homogenates by centrifugation for 20 hours at 33,000 rpm on a cesium chloride gradient (5.7 M CsCl and 100 mM EDTA). After centrifugation RNA pellets were resuspended in 300 μl TE (10 mM Tris, 1 mM EDTA) containing 0.1% SDS, precipitated with 3 M sodium acetate (0.1 vol) and ethanol (3 vol) and stored at −80°C prior to analysis. EPO mRNA was measured by RNAse protection, as described [5], using a rat EPO probe containing 132 base pairs of exon V and approximately 300 base pairs of the adjoining intron inserted into pSP 64 for generation of RNA transcripts. Transcripts were continuously labeled with alpha ^{32}P-GTP (410 Ci/mmol; Amersham International). For hybridization total RNA was dissolved in buffer (80% formamide, 40 mM PIPES, 400 mM sodium chloride, 1 mM EDTA, pH 8) and the RNA concentration determined by measurement of optical density at 260 nm. The concentration was adjusted to yield 50 μl samples containing 100 μg RNA. Hybridization was performed overnight at 60°C with 0.5 to 1 × 10^6 cpm radiolabeled EPO probe. RNAse digestion with RNAse A and T1 was carried out at 20°C for 30 minutes and terminated by the addition of proteinase K and SDS prior to purification of the protected fragments by phenol/chloroform extraction, ethanol precipitation and electrophoresis on a denaturating 10% polyacrylamide gel. After autoradiography of the dried gel at −70°C protected EPO mRNA bands were excised from the dried gel and counted using a flat bed liquid scintillation counter (1205; Betaplate™ Pharmacia-Wallac OY, Turku, Finland). The number of counts per minute obtained from each EPO mRNA sample was divided by the quantity of total RNA analyzed and expressed relative to an external standard. The standard consisted of 5 μg pooled RNA extracted from the kidneys of severely anemic rats, which was run with each assay and assigned an arbitrary value of 1.0. The yield of total RNA extracted from known amounts of tissue was used to calculate the concentration of EPO mRNA (in arbitrary units) per gram kidney tissue.

Determination of serum EPO levels

Serum EPO concentrations were determined by radioimmunoassay as described [22] with the use of a rabbit antiserum raised against pure recombinant human EPO and iodinated human EPO (Amersham Int.) as tracer. A rat serum pool enriched in EPO was prepared by exposing donor animals to hypoxia and was used as standard, as described [4, 6, 15, 18], after calibration against the II. International Reference Preparation by *in vivo* bioassay [22].

Statistics

Student's unpaired *t*-test was used for comparison of groups and analysis of variance to determine the significance level of linear regressions. $P < 0.05$ was considered significant.

Results

EPO production under graded degrees of hypoxia

Figure 1 illustrates changes in serum EPO concentrations (left) and renal EPO mRNA levels (right) in rats exposed to different oxygen tensions for eight hours. Both parameters increased exponentially with decreasing oxygen tensions. Under the most severe stimulus applied (7.5% O_2) EPO mRNA concentrations in kidneys increased approximately three hundredfold [0.91 ± 0.1 (20% O_2) vs. 274 ± 10 (7.5% O_2); mean ± SE, $N = 3$], while serum hormone concentrations rose approximately one hundredfold [23.7 ± 1.4 (20% O_2) vs. 2105 ± 298 (7.5% O_2); mean ± SE, $N = 3$].

In situ hybridization for EPO was performed on one kidney of each of the animals studied. Cells containing EPO mRNA were detectable using an antisense rat EPO probe, whereas no specific hybridization was detected when the sense strand derived from the rat EPO cDNA was used as a probe. Under all experimental conditions, irrespective of the severity of hypoxia, an accumulation of silver grains, indicating the presence of EPO mRNA, was exclusively detected over cells located in the peritubular space, as demonstrated in Figure 2. This figure shows the view of the same area of kidney cortex of one of the animals in conventional throughlight (A) and under epipolarization (B), and thereby illustrates the intensity of specific signals as compared to background radioactivity. No positive signals were detected over glomerular or tubular cells.

Figure 3 shows low magnification and Figure 4 higher magnification photomicrographs, illustrating the results of in situ hybridization on sections from animals studied under different conditions: a normoxic rat (Fig. 4E) and rats exposed for eight hours to 11.5% O_2 (Fig. 3A and 4A), 9% O_2 (Figs. 3B and 4B) or 7.5% O_2 (Fig. 3C and 4C). To aid identification of positive hybridization signals, circles are used on these figures to mark single cells or clusters of few cells covered with silver grains,

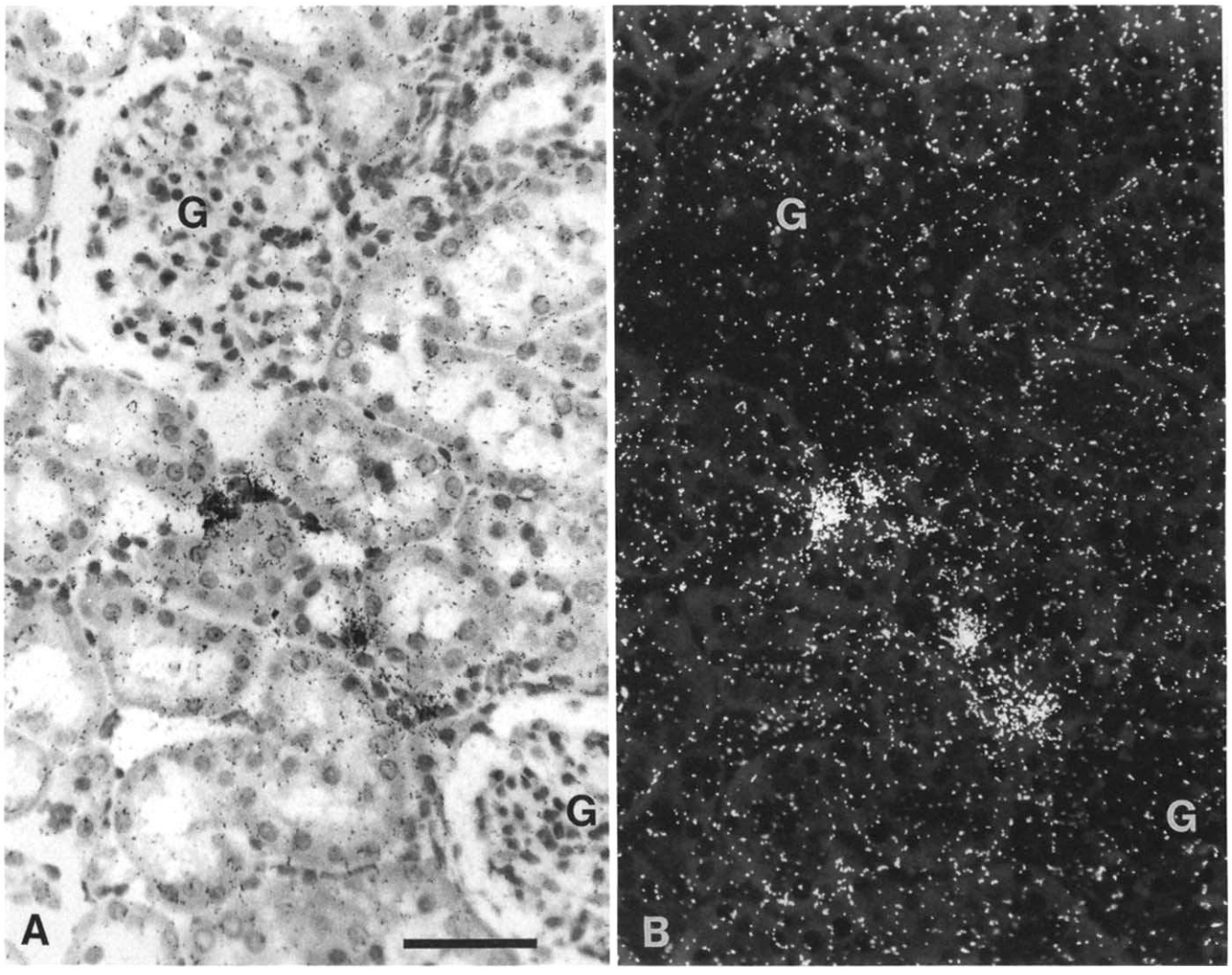

Fig. 2. *Photomicrographs of an in situ autoradiogram showing the presence of silver grains over peritubular cells after hybridization with an antisense rat EPO cRNA probe.* **(A)** The view using conventional throughlight microscopy, and **(B)** using epipolarization. Note the marked difference in intensity between "positive" signals and background radioactivity. No hybridization signals were found over tubular or glomerular cells. G, Glomerulus. Final magnification 380-fold. Bar = 50 μm.

which were all identified by high magnification microscopy of each section.

Irrespective of the severity of hypoxia, the location of peritubular cells containing EPO mRNA did not vary with respect to two criteria. First, EPO producing cells were only located in the renal cortex and no signals were found over cells in the medulla (not shown). Second, as illustrated in Figure 3, within the renal cortex, EPO producing cells were almost exclusively present in the cortical labyrinth and only few single cells were occasionally detected in the medullary rays. Depending on the severity of hypoxia, significant changes were apparent, however, in the number of cells containing EPO mRNA. Under normoxic conditions single cells containing EPO mRNA or clusters of few cells (Fig. 4E) were present in deep cortical regions (not shown). Under hypoxic stimulation of low (11.5% O_2) and intermediate (9% O_2) severity an increasing number of cells producing EPO mRNA was found in deep- and midcortical areas (Fig. 3A and B). A further marked increase in the number of cells containing EPO mRNA occurred after severe stimulation (7.5% O_2; Fig. 3C) and under this condition the distribution of signals extended to subcapsular tissue.

For quantitative comparison of the number of EPO producing cells with renal tissue concentrations of EPO mRNA, the number of positively labeled cells per square cm of renal cortex was counted in specimens of each kidney. Care was taken to prepare sections from the center of the kidney in identical transverse orientation in all animals studied. Figure 5 demonstrates that the mean number of EPO producing cells per unit area of kidney cortex was exponentially related to oxygen tensions in a manner very similar to that observed for renal tissue concentrations of EPO mRNA (Fig. 1B). Furthermore, as shown in Figure 6, comparison of the number of EPO producing cells and renal EPO mRNA concentrations in individual animals revealed a near linear relationship. When comparing only normoxia and severe hypoxia (7.5% O_2), however, the mean increase in renal EPO mRNA concentrations was slightly

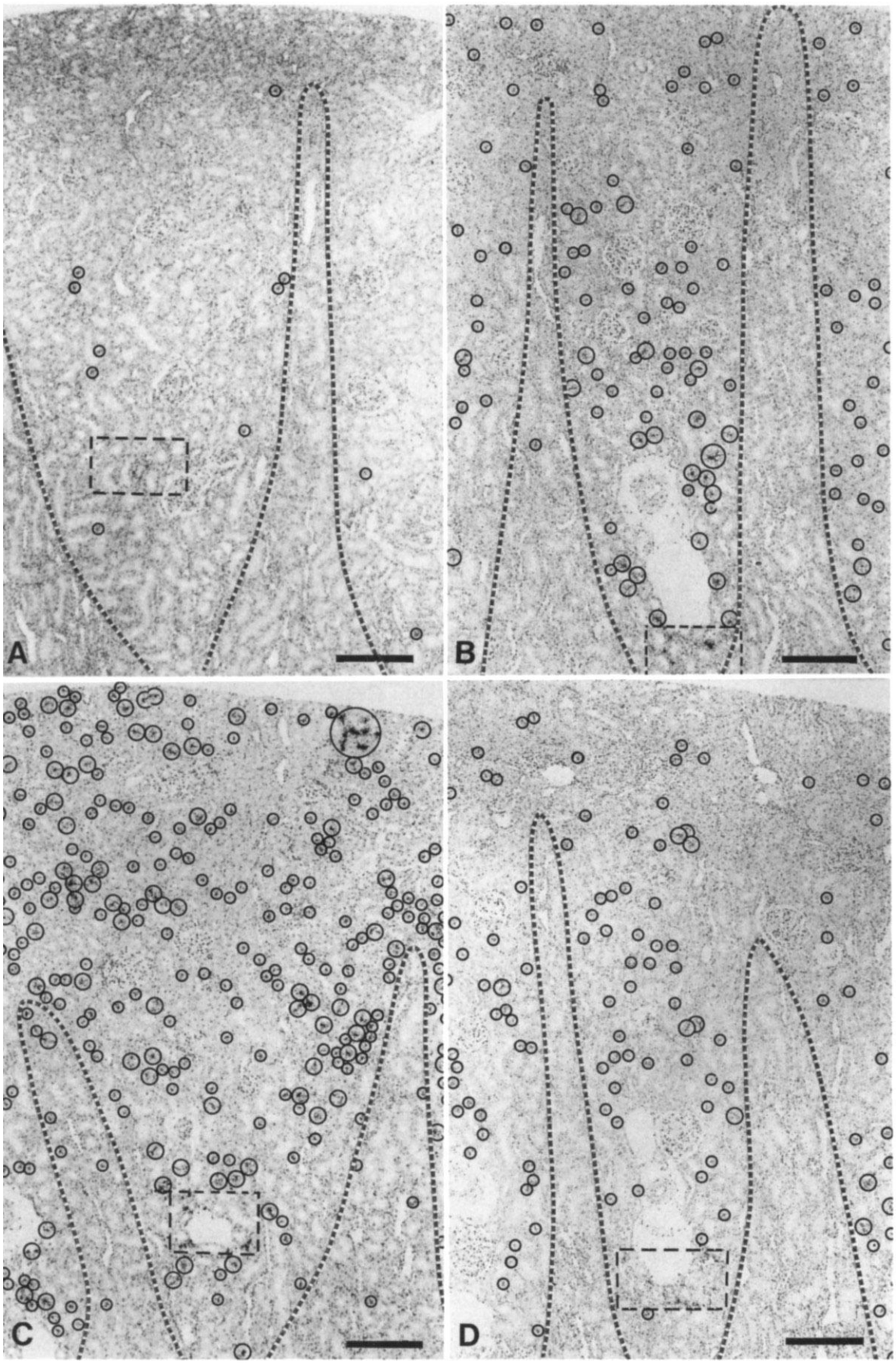

Fig. 3. *Photomicrographs of in situ autoradiograms showing the localization of cells producing EPO mRNA in the renal cortex of rats exposed to* **(A)** *11.5% O_2 for 8 hours,* **(B)** *9% O_2 for 8 hours,* **(C)** *7.5% O_2 for 8 hours,* **(D)** *7.5% O_2 for 32 hours.* Single cells or clusters of few cells covered with silver grains were identified by high magnification microscopy and marked on photomicrographs with circles. Dotted lines indicate border between cortical labyrinth and medullary rays. High magnification photomicrographs of the outlined rectangular areas are given in Figure 4. Final magnification 72-fold. Bar = 200 μm.

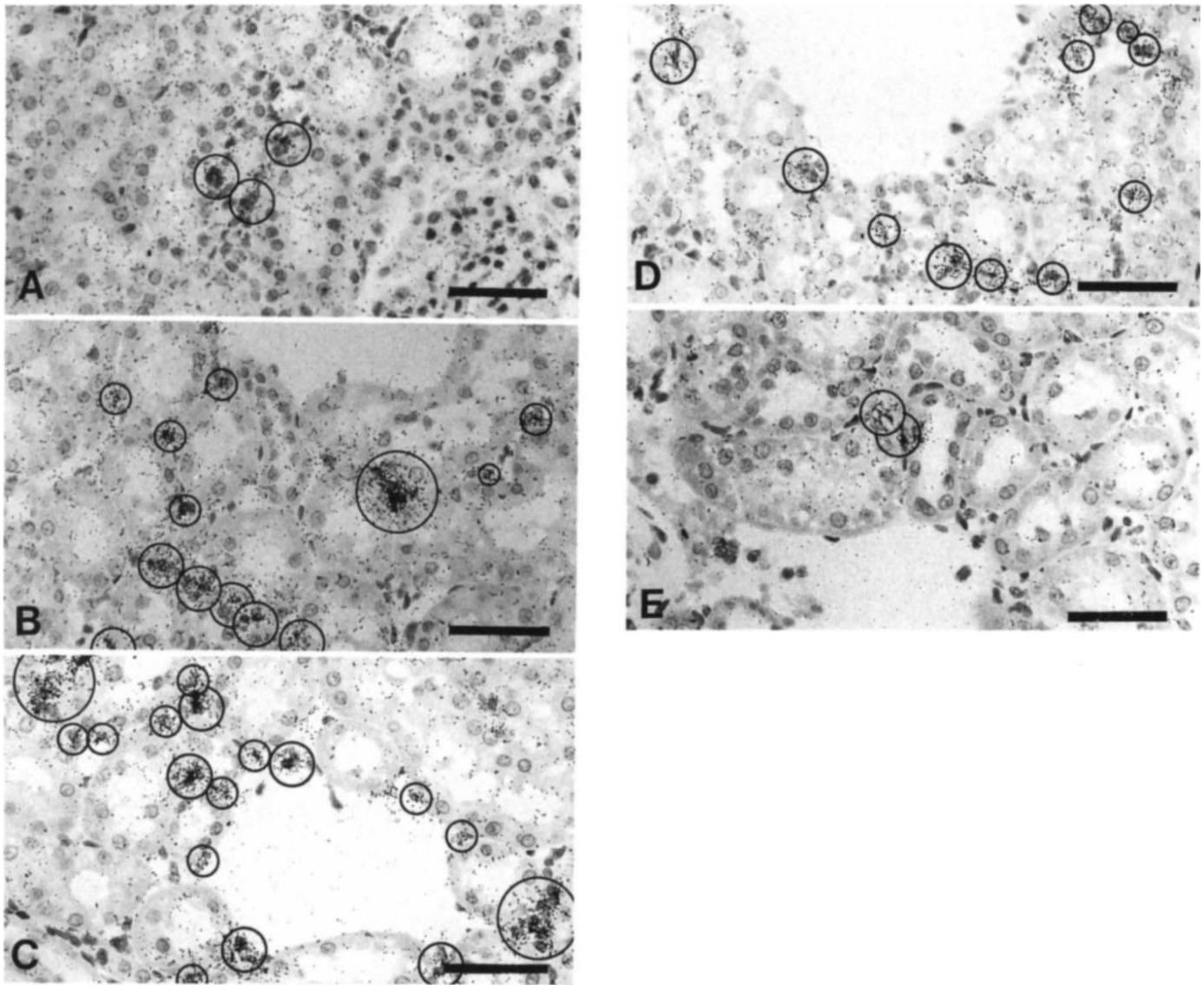

Fig. 4. *High magnification photomicrographs of in situ autoradiograms of kidney sections illustrating the location of EPO-producing cells in deep cortical areas.* **(A–D)** Photomicrographs of the same autoradiograms shown in Figure 3, corresponding to the rectangles outlined in Figure 3 (A = 11.5% O_2 for 8 hours, B = 9% O_2 for 8 hours, C = 7.5% O_2 for 8 hours, D = 7.5% O_2 for 32 hours). **(E)** Photomicrograph of an in situ autoradiogram of a kidney section from a normoxic animal. Cells covered with silver grains are marked with circles. Final magnification 290-fold. Bar = 50 μm.

higher (296-fold) than the mean increase in the number of cells containing EPO mRNA (201-fold), and although we did not precisely quantitate the intensity of hybridization signals, the grain density over single cells appeared somewhat higher under severe hypoxia (7.5% O_2) than under normoxia or mild hypoxia (11.5% O_2) (Fig. 4A, C and E).

EPO production after different periods of hypoxic exposure

The mean serum EPO level of animals exposed to 7.5% oxygen for four hours was 403.7 ± 39.7 mU/ml (± SE, N = 3) and that is approximately 20% of the value observed after eight hours of exposure to the same stimulus [2105 ± 298 mU/ml (mean ± SE, N = 3), Fig. 1]. As expected from earlier investigations [1, 13–15], more prolonged hypoxic exposure resulted in decline of the serum hormone concentration and after 32 hours the mean serum EPO level was only about 50% of the value measured after eight hours of hypoxia (1018.8 ± 256.6 mU/ml; mean ± SE, N = 6). As shown in Figure 7 (left), these variations in serum EPO concentrations were paralleled by changes in renal EPO mRNA. EPO mRNA levels at 4 and 32 hours amounted to 26% and 47%, respectively, of those values measured after eight hours of hypoxic exposure. The distribution of EPO producing cells, however, as determined by *in situ* hybridization, was very similar after 4 and 8 hours (not shown), and their mean numbers after 4 hours and after 8 hours (Fig. 7, right) were not statistically different. The ratio of tissue concentrations of EPO mRNA to the number of EPO producing cells, which provides an approximate estimate of the EPO mRNA content of single EPO producing cells, was threefold higher in animals after 8 hours than after 4 hours of hypoxic

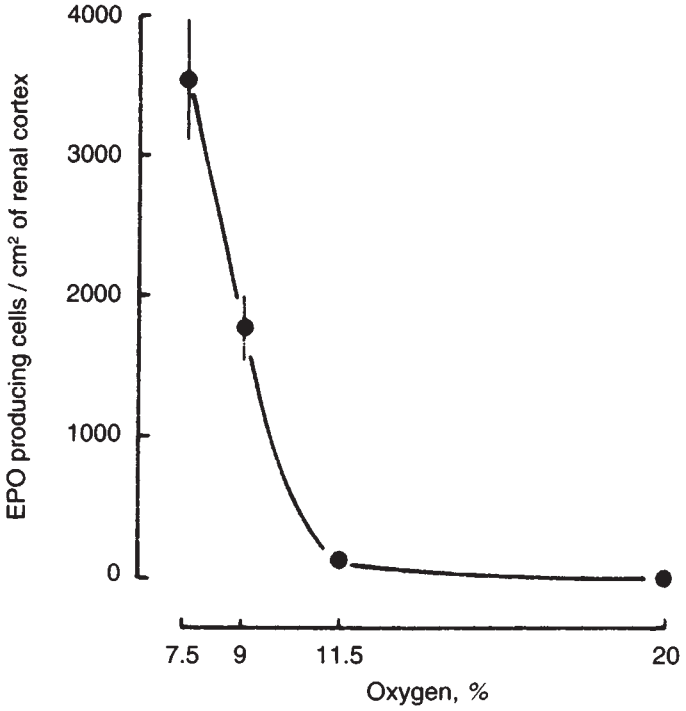

Fig. 5. *Relationship between the number of EPO producing cells per cm² of renal cortex and oxygen concentrations in animals studied under normoxic conditions or exposed to different degrees of hypoxia (7.5% O_2, 9% O_2, 11.5% O_2) for eight hours.* EPO producing cells were counted twice in independent hybridization experiments on in situ autoradiograms of sections from three animals in each group. Values are mean ± SE of the average number of counts per animal for three animals.

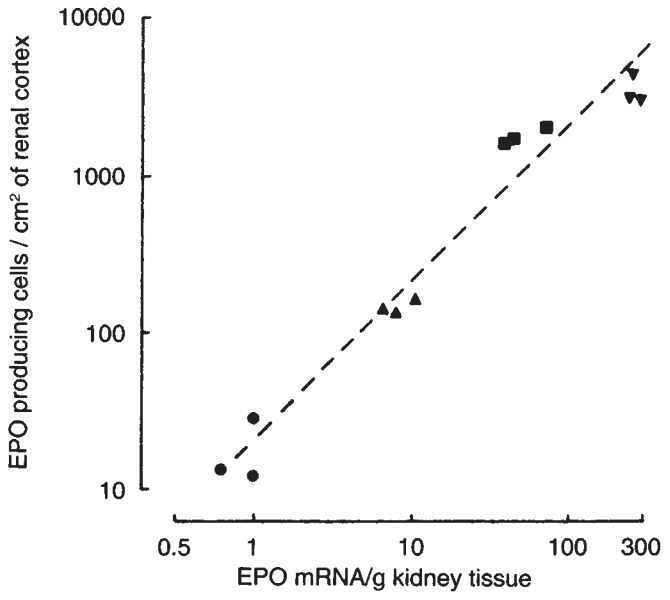

Fig. 6. *Relationship between the number of EPO producing cells per cm² of renal cortex in one kidney and EPO mRNA concentrations in the contralateral kidney in individual animals studied under normoxic conditions or after 8 hours exposure to hypoxia of different severity.* Symbols are: (●) 20% O_2; (▲) 11.5% O_2; (■) 9% O_2; (▼) 7.5% O_2. The number of EPO producing cells correlated linearly with tissue concentrations of EPO mRNA: log (EPO-producing cells/cm² of renal cortex) = 1.31 + log (EPO mRNA/g kidney tissue) × 0.98; $r^2 = 0.96$, $P < 0.0001$.

exposure. In contrast, after 32 hours of hypoxia there were concurrent changes in renal EPO mRNA levels and the number of EPO producing cells (Fig. 7A and B), with a slightly greater reduction in the latter (63%) than in the former (53%). Figure 3D illustrates, that EPO producing cells after 32 hours of hypoxic exposure disappeared mainly from superficial cortical areas, so that remaining cells were predominantly located in deep and midcortical regions. Under all time points investigated no difference was observed with respect to the peritubular location of EPO producing cells in the cortical labyrinth (Fig. 4D).

Discussion

This study aimed to define the location and distribution of EPO producing cells in the kidney under conditions of reduced arterial oxygen tensions and to compare them with previous observations in anemic animals. In contrast to what might be expected from differences in the mode of reduction in oxygen availability and in the quantity and the kinetics of EPO formation under anemic and hypoxic hypoxia, our results indicate that the intrarenal cellular pattern of EPO gene expression under anemic and hypoxic hypoxia is strikingly similar.

First, the localization of cells accumulating EPO mRNA appears virtually identical under both anemic and hypoxic hypoxia, since *in situ* hybridization in the present investigation showed EPO producing cells exclusively in the peritubular space of the renal cortex (Figs. 2 to 4), as has been previously reported in anemic mice [8, 9, 19], anemic rats [10] and human polycystic kidneys [11]. An observation made for the first time in this study was that within the renal cortex, EPO producing cells were located almost exclusively in the cortical labyrinth, whereas only few cells containing EPO mRNA were found in the medullary rays (Fig. 3). Although, as in earlier studies, *in situ* hybridization did not allow identification of the cell type within the cortical interstitium producing EPO, it appears highly likely in view of our results, that cell specific expression of the EPO gene is independent of the mode of limitation of oxygen supply. These findings are in contrast, however, to those of Maxwell et al [12], who reported EPO gene expression in tubular cells during hypoxic hypoxia, but, as discussed previously [23], the use of ^{32}P-labeled oligonucleotide probes on 10 μm sections by these investigators may be less favorable for resolving EPO gene expression at the cellular level.

Second, when graded degrees of hypoxic hypoxia were applied for an identical time period, the predominant control of EPO mRNA levels was found to operate through the number of cells expressing the EPO gene. In normoxic animals a few clusters of EPO producing cells were seen in deep cortical areas. With decreasing oxygen tensions more cells were recruited in these areas and recruitment also extended outwards to midcortical tissue (Fig. 3A and B). Positive cells were observed throughout the renal cortex following severe stimulation (Fig. 3C). This pattern of recruitment is very similar to that previously reported in mice subjected to different severities of anemia [19]. The overall number of cells detected in kidney tissue by *in situ* hybridization for EPO mRNA increased exponentially with decreasing oxygen tensions (Fig. 5), and under the most severe stimulus tested, approximately 200-fold more EPO producing cells were detectable per unit area of

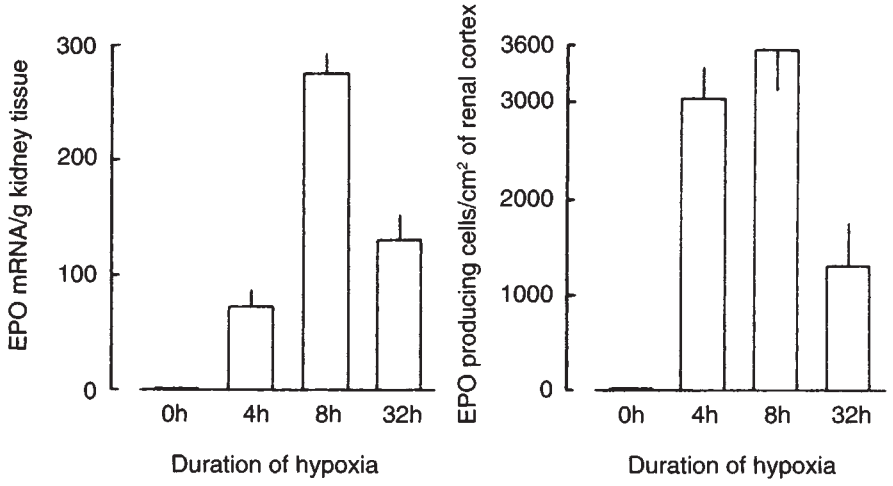

Fig. 7. *Time dependent changes in renal EPO mRNA concentrations* (**A**) *and the number of EPO producing cells per cm² of renal cortex* (**B**) *in rats exposed to 7.5% O_2 for up to 32 hours.* Values are mean ± SE, $N = 3$ at 0 hr, 4 hr and 8 hr and 6 at 32 hr. EPO mRNA concentrations after 4 hr and 32 hr were significantly different from those after 8 hr of hypoxic exposure. The numbers of EPO producing cells were significantly different after 8 and 32 hours of hypoxia but not significantly different after 4 and 8 hours of hypoxia.

kidney cortex than in normoxic animals. Moreover, as in anemic mice [19], under different degrees of hypoxia a near linear relationship was observed between the number of EPO producing cells detected on sections of one kidney and the total organ quantity of EPO mRNA measured in the contralateral kidney (Fig. 6). With increasing severity of hypoxia, however, the slope of this correlation tended to flatten somewhat, which may indicate an increasing amount of EPO mRNA in single cells. However, when considering the recruitment of EPO producing cells within the cortical labyrinth (Fig. 3), a deviation from linearity between the number of EPO producing cells in a planar section and tissue concentrations of EPO mRNA may also arise for geometrical reasons. The three dimensional structure of the cortical labyrinth can be approximated as a cone with its tip at the corticomedullary border and the base at the outer kidney surface. With increasing distance from the tip of a cone its volume increases at higher proportion than the area of a longitudinal section. Since *in situ* hybridization determines the number of EPO producing cells in two dimensions on longitudinal sections and EPO mRNA levels in tissue homogenate reflect the amount of EPO RNA per unit volume, the relationship between both parameters does not only depend on the amount of EPO mRNA per cell, but also on the extent of recruitment. Thus, although a major factor responsible for the increase in total organ EPO mRNA with increasing severity of hypoxia is undoubtedly an increase in the number of cells accumulating EPO mRNA, an additional increase in EPO mRNA concentrations of single cells is possible, although it can not be quantitated precisely under these conditions.

Taken together, our observations suggest that the basic mechanisms through which renal EPO formation is increased when either arterial oxygen tension or oxygen carrying capacity decreases are largely the same. The mechanisms directing expression of the EPO gene to more superficial areas of the renal cortex with increasing severity of both types of hypoxia, however, remain speculative. Observations in human hepatoma cells have provided evidence that oxygen sensing and EPO production can reside in the same cell [24], and it was recently shown that the distribution of cells producing EPO in livers of anemic transgenic mice corresponds to known oxygen gradients [20]. Whether local oxygen tensions determine the distribution of EPO gene expression in the kidney is, however, less clear. Although studies in isolated perfused kidneys have shown, that oxygen sensing mechanisms are located in the kidney itself [5, 6], it remains unknown whether renal EPO producing cells directly respond to changes in their oxygenation. Furthermore, although several studies have shown that oxygen gradients exist in the renal cortex [25–27], their distribution has not been established sufficiently to allow a direct comparison with the pattern of EPO gene expression. It is noteworthy, however, that deep and midcortical regions of the labyrinth, that is, those areas where EPO producing cells are found under mild and moderate hypoxia, are in part supplied by blood which has passed through medullary rays and its oxygen content may thus be lower than that in capillaries of the superficial cortex, which directly emerge from efferent glomerular vessels [28]. The observation that EPO producing cells are primarily present in the cortical labyrinth and not the medullary rays, also rises possibility that the neighborhood of distinct nephron segments, that is, segments of convoluted tubules, is an important determinant for oxygen dependent expression of the EPO gene in peritubular cells. This would be compatible with evidence suggesting that transport activity of the proximal tubule influences EPO formation [29], and that in anemic mice EPO producing cells were primarily found adjacent to proximal tubular cells [30].

Comparison of the total EPO mRNA and the cellular distribution of EPO mRNA as determined by *in situ* hybridization also provides some insight into the mechanisms underlying the time course of the EPO production in response to hypoxic hypoxia. Our results indicate that the same number of cells that express the gene when EPO production is approximately maximal, after eight hours, is already activated after four hours of hypoxia (Fig. 7) and the fourfold increase in total renal EPO mRNA occurring between four and eight hours thus appears mainly due to an increased accumulation of EPO mRNA in single cells. This would be consistent with the assumption that local oxygen concentrations determine the distribution of EPO producing cells, since tissue oxygen tension should drop to rather constant values soon after onset of hypoxia. The time dependent increase in EPO mRNA levels of single cells, as observed between four and eight hours of hypoxia, could occur despite constant cellular oxygen supply as a result of time dependent signal transduction, transcriptional activation and

possibly changes in EPO mRNA metabolism [1]. Upon more prolonged hypoxic exposure, renal EPO mRNA levels declined again, as previously reported [15], and after 32 hours mean EPO mRNA concentration amounted to only 47% of the concentration achieved after eight hours exposure to the same hypoxic stimulus (7.5% O_2; Fig. 7). This reduction in EPO mRNA levels appeared to result from "reversed" recruitment of EPO producing cells, so that the ratio between tissue concentrations of EPO mRNA and cell number did not change significantly. Cells containing EPO mRNA disappeared mainly from superficial cortical areas and thus the pattern of EPO gene expression after 32 hours exposure to 7.5% O_2 (Fig. 3D) resembled the picture seen after eight hours of exposure to less severe hypoxia (9% O_2, Fig. 3B). This observation would be compatible with the hypothesis that renal oxygenation is improved during more prolonged hypoxia, such as through a rightward shift in the oxygen binding curve [1]. Alternatively, it is also possible that renal oxygenation is unchanged, but that adaptation occurs at the level of EPO producing cells, such that a putative "oxygen-threshold" for activation of the EPO gene is decreased in all potentially EPO producing cells. The local oxygen tension might then only be low enough to maintain EPO gene expression in those areas of the renal cortex where EPO production also occurs under less severe stimulation.

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Note added in proof

Since this work was submitted, evidence has been obtained that the peritubular cells expressing EPO mRNA are fibroblasts [31].

References

1. Jelkmann W: Erythropoietin: Structure, control of production, and function. *Physiol Rev* 72:449–489, 1992
2. Bondurant MC, Koury MJ: Anemia induces accumulation of erythropoietin mRNA in the kidney and liver. *Mol Cell Biol* 6:2731–2733, 1986
3. Schuster SJ, Wilson JH, Erslev AJ, Caro J: Physiologic regulation and tissue localization of renal erythropoietin messenger RNA. *Blood* 70:316–318, 1987
4. Eckardt K-U, Ratcliffe PJ, Tan CC, Bauer C, Kurtz A: Age dependent expression of the erythropoietin gene in rat liver and kidneys. *J Clin Invest* 89:753–760, 1992
5. Ratcliffe PJ, Jones RW, Phillips RE, Nicholls LG, Bell JI: Oxygen-dependent modulation of erythropoietin mRNA levels. *J Exp Med* 172:657–660, 1990
6. Scholz H, Schurek HJ, Eckardt K-U, Kurtz A, Bauer C: Oxygen dependent erythropoietin production by the isolated perfused rat kidney. *Pflügers Arch* 418:228–233, 1991
7. Pagel H, Jelkmann W, Weiss CH: Isolated serum-free perfused rat kidneys release immunoreactive erythropoietin in response to hypoxia. *Endocrinology* 128:2633–2638, 1991
8. Koury ST, Bondurant MC, Koury MJ: Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. *Blood* 71:524–527, 1988
9. Lacombe C, Da Silva J-L, Bruneval P, Fournier J-G, Wendling F, Casadevall N, Camilleri J-P, Bariety J, Varet B, Tambourin P: Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. *J Clin Invest* 81:620–623, 1988
10. Schuster SJ, Koury ST, Bohrer M, Salceda S, Caro J: Cellular sites of extrarenal and renal erythropoietin production in anemic rats. *Br J Haematol* 81:153–159, 1992
11. Eckardt K-U, Möllmann M, Neumann R, Brunkhorst R, Burger HU, Lonnemann G, Scholz H, Keusch G, Buchholz B, Frei U, Bauer C, Kurtz A: Erythropoietin in polycystic kidneys. *J Clin Invest* 84:1160–1166, 1989
12. Maxwell AP, Lappin TRJ, Johnston CF, Bridges JM, McGeown M: Erythropoietin production in kidney tubular cells. *Br J Haematol* 74:535–539, 1990
13. Abbrecht PH, Littell JK: Plasma erythropoietin in men and mice during acclimatization to different altitudes. *J Appl Physiol* 32:54–58, 1972
14. Fried W, Johnson C, Heller P: Observations on the regulation of erythropoiesis during prolonged periods of hypoxia. *Blood* 36:607–616, 1970
15. Eckardt K-U, Dittmer J, Neumann R, Bauer C, Kurtz A: Decline of erythropoietin formation at continuous hypoxia is not due to feedback inhibition. *Am J Physiol* 258:F1432–F1437, 1990
16. Reynafarje C, Ramos J, Faura J, Villavicencio D: Humoral control of erythropoietic activity in man during and after altitude exposure. *Proc Soc Exp Biol Med* 116:649–650, 1964
17. Seferynska I, Brookins J, Rice JC, Fisher JW: Erythropoietin production in exhypoxic polycythemic mice. *Am J Physiol* 256:925–929, 1989
18. Eckardt K-U, Le Hir M, Tan CC, Ratcliffe PJ, Kaissling B, Kurtz A: Renal innervation plays no role in oxygen dependent control of erythropoietin mRNA levels. *Am J Physiol* 263:F925–F930, 1992
19. Koury ST, Koury MJ, Bondurant MC, Caro J, Graber SE: Quantitation of erythropoietin-producing cells in kidneys of mice by in situ hybridization: Correlation with hematocrit, renal erythropoietin mRNA, and serum erythropoietin concentration. *Blood* 74:645–651, 1989
20. Koury ST, Bondurant MC, Koury MJ, Semenza GL: Localization of cells producing erythropoietin in murine liver by in situ hybridization. *Blood* 77:2497–2503, 1991
21. Cox KH, Deleon DV, Angerer LM, Angerer RC: Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Devel Biol* 101:485–502, 1984
22. Eckardt K-U, Kurtz A, Hirth P, Scigalla P, Wieczorek L, Bauer C: Evaluation of the stability of human erythropoietin in samples for radioimmunoassay. *Klin Wochenschr* 66:241–245, 1988
23. Koury ST, Bondurant MC, Koury MJ: Correspondence (reply): The site of erythropoietin production: localization of erythropoietin mRNA by radioactive in situ hybridization. *Blood* 78:2469–2471, 1991
24. Goldberg MA, Glass GA, Cunningham JM, Bunn HF: The regulated expression of erythropoietin by two human hepatoma cell lines. *Proc Natl Acad Sci USA* 84:7972–7976, 1987
25. Aukland K, Krog J: Renal oxygen tension. *Nature* 188:671, 1960
26. Baumgärtl H, Leichtweiss HP, Lübbers DW, Weiss C, Huland H: The oxygen supply of the dog kidney: Measurements of intrarenal pO_2. *Mirovasc Res* 4:247–257, 1972
27. Schurek HJ, Jost U, Baumgärtl H, Bertram H, Heckmann U: Evidence for a preglomerular oxygen diffusion shunt in rat renal cortex. *Am J Physiol* 259:F910–F915, 1990
28. Kriz W, Kaissling B: Structural organization of the mammalian kidney, in *The Kidney: Physiology and Pathophysiology*, edited by Seldin DW, Giebisch G, New York, Raven Press, 1992, pp. 707–778
29. Eckardt K-U, Kurtz A, Bauer C: Regulation of erythropoietin formation is related to proximal tubular function. *Am J Physiol* 256:F942–F947, 1989
30. Koury ST, Thamer SL, Koury MJ, Bondurant MC: Erythropoietin-producing interstitial cells are primarily found adjacent to proximal tubules in murine kidneys. (abstract) *Blood* 74 (Suppl 1):18a, 1989
31. Bachmann S, LeHir M, Eckardt K-U: Colocalization of erythropoietin mRNA and ecto-5′-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. *J Histochem Cytochem* (in press)